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AUTORADIOGRAPHY WITH PLANT TISSUE[1]

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INTRODUCTION

Several terms have been used to describe the photographic results obtained after an object containing radioactivity is placed in contact with, or near to, a photographic emulsion. Tauxe et al. (175) offer acceptable reasons for use of the term "autoradiograph" to designate the technique, and the term "autoradiogram" to designate the result of the technique. The usage of these terms, although questioned by some workers (117), is generally accepted and is employed in this review.

[1] Florida Agricultural Experiment Station Journal Series, No 604.

The historical development of autoradiography has been amply discussed in two books on the subject (43, 197), and in review articles presented in the past 14 years (12, 25, 35, 40, 53, 58, 59, 77, 90, 91, 92, 95, 98). These reviews have in general been concerned with techniques, applicable to biological tissue other than plants. This is understandable when it is considered that the majority of investigators using the technique have been concerned with animal tissue. In all cases where the techniques have been applied to plant materials, modifications of methods previously developed with animal tissue have been employed. Several analytical investigations of the application of autoradiography to plants have been attempted (64, 133, 167). In these reports it is apparent that the method is more difficult to apply to plant material for several reasons: *a*) preparation of plant material for histological investigation takes longer than for animal tissue; *b*) plant cells, in general, are larger, and the vacuoles easily lose the radioisotopes to solutions used in the autoradiographic process.

Autoradiography has proved especially useful in the efforts of biologists to locate and to interpret chemical and anatomical relationships within an organism. The current objective in refinement of technique is to increase resolution in the autoradiographic image, and thus increase its usefulness in locating small intracellular deposits or accumulations of radioactive material.

This article is an attempt to summarize the information available in the literature that pertains to the application of autoradiography to plants and to discuss some of the results obtained from autoradiograms.

PHOTOGRAPHIC EMULSION AS A MEASURING INSTRUMENT AND TERMINOLOGY

Trimmer (180) describes autoradiography as a digital type analytical measurement. Such measurements consist of the taking of a census; in autoradiography a numerical density of radioactive nuclides is observed. Density alone does not adequately describe all the information revealed in an autoradiogram. Trimmer suggests the term "density-structure" class of measurement to define autoradiography. The direct count is the number of developed spots on a photographic film. This is interpreted as the number of radioactive atoms associated with the material under observa-

tion. Boyd (43) compares the photographic emulsion with other types of instruments used to record radioactivity.

Axelrod (12) discusses autoradiography under two general types, namely whole tissue or gross autoradiograms, and histological autoradiograms, whereas Wainwright (186) defines these as survey and detail autoradiograms. The gross or survey autoradiogram is the result obtained when an entire plant or plant organ containing a radioisotope is placed in contact with a photographic emulsion which is exposed and later developed to reveal the general distribution of the isotope in the organ. The detail or histological autoradiogram is obtained by bringing a photographic emulsion into close contact with a thin section (1 μ–50 μ) containing the radioisotope. In the latter case microscopic examination of the autoradiogram is essential to correlate developed emulsion grains with the tissues of the histological section.

Several terms used in the discussion of autoradiography need to be defined. They are:

EMULSION: A thin layer of gelatin in which silver halide crystals are embedded. This sensitive layer may be deposited on glass or plastic, attached to a strippable acetate backing, or obtained in liquid or pellicle form.

LATENT IMAGE: Any irradiation to which the emulsion is sensitive will render the silver halide crystal developable. The crystals, that are excited in this way, form the latent image.

FOG, BACKGROUND or ARTIFACT: Silver halide crystals may be rendered developable by some means other than the passing of a proton, beta particle or alpha particle through them. Such crystals will "develop out" in the same fashion as the "true" latent image and interfere with the final analysis.

RESOLUTION or RESOLVING POWER: Smallest distance between two developed grains yet still recognized as distinct particles (77).

SPEED or SENSITIVITY: The quanta or irradiance a silver halide crystal must absorb to form a latent image that is developable under a given condition of exposure and processing (24, 58).

DEVELOPER: The chemical solution used to reduce the silver halide crystals on which a latent image has been produced.

FIXING SOLUTION: The chemical solution used in the photographic process to remove the silver halide crystals that have not been reduced by the developer.

PHYSICAL PRINCIPLES

Latent Image Formation: Development and Factors Influencing Its Retention in the Photographic Emulsion

Gomberg (87) and Boyd (43) discuss the theory of latent image formation in terms of autoradiography. According to Gomberg (87), this theory developed by Gurney and Mott in 1938 brought together the several known details about the photographic process that had been previously shown by other workers. These are:

a) Identification of sensitivity specks on the silver halide grains. Crystals of silver halide are not perfect; on the surface or in the crystal will appear silver sulfide molecules that are not a part of the crystal lattice. These imperfections are the sensitivity specks.

b) The image formed in the emulsion is metallic silver.

c) The conductivity of silver halide is electrolytic, caused by the mobility of ions and the migration of silver ion lattice site vacancies. The conductivity of silver halide is a function of the temperature.

d) The existence of photoelectrons in the crystals. This is independent of temperature.

e) The sensitivity specks are the only points on the crystals where silver is deposited regardless of the nature of irradiation.

f) The quantum efficiency of the photographic process approaches one.

The formation of a latent image is believed to occur in the following manner:

a) Photons or other forms of energy capable of producing ion pairs are captured by a bromide ion in the lattice.

b) The electron produced ($Br \rightarrow Br + e^-$) moves through the conducting band to a sensitivity speck which acts as a trap.

c) The neutral bromide atom diffuses out of the crystal.

d) Silver ions now migrate to the sensitivity speck by electrostatic force. On arrival at the trap the silver ion is neutralized and the process is repeated many times.

e) A silver deposit accumulates, and when large enough, becomes the latent image.

The development of the latent image, even though not completely understood, is thought to proceed in the following way:

a) The developing solution reduces the silver halide of the crystal. Reduction of the silver occurs very slowly if a latent image has not been formed. On the crystals where an image has been produced the reduction is rapid. The latent image acts as a catalyst for the chemical reduction.

b) The metallic silver is deposited in a filament that grows around the crystal. If development proceeds long enough the black metallic silver deposit becomes visible to the unaided eye.

c) Silver halide crystals that have no latent image are removed by the action of the thiosulfate ion in the fixative bath.

The loss of the latent image after it is formed is equivalent to the loss of sensitivity. In the end result it would be impossible to distinguish between the two (165). The factors which contribute to these phenomena are:

a) The moisture content of the emulsion (17). The rate of latent image fading is an exponential function of the quantity of water retained by the gelatin (16).

b) The temperature of the emulsion. Fading of the latent image is an exponential function of the reciprocal of the temperature.

c) The storage atmosphere. Latent image fading is twice as great in oxygen as in air, and ten times as fast as in nitrogen (17).

d) Hydrogen ion concentration. The lower the pH of the emulsion, the more rapid the loss of latent image.

e) The exposure time. If the exposure time is prolonged, the probability of latent image loss increases.

f) The type of photographic emulsion. Sensitivity and latent image loss are more prevalent in fine grain emulsions, such as those used in nuclear track recording (16).

g) The nature of the radiation. The lower the energy associated with the nuclear particle, the more rapid will be the latent image fading.

Often it is desirable to remove latent images from nuclear emulsions prior to their use. This has been accomplished by use of water vapor (3, 17, 46) and H_2O_2 (197). Intensifying underexposed autoradiograms has also been accomplished (55) for X-ray film.

Photographic Emulsions for Autoradiography

There are several types of photographic emulsion available for autoradiography. The choice of emulsion depends on the nuclear radiation, the biological material and the type of autoradiograms sought. For gross or survey autoradiography no-screen X-ray film is often used. X-ray film is more sensitive to irradiation than nuclear emulsions (77, 168). At the same time, the resolution of the X-ray film is poor and the background is high (43). It takes about ten times as many disintegrations from C^{14} per square centimeter of film to give a density of 0.6 in NTB nuclear emulsion as in X-ray emulsion (49). On the other hand, it has been shown (168) that the optical densities obtained from equal flux of ionizing particles of various energies are quite different. Photographic emulsions that have been used in autoradiography include the medium lantern plates, type M stripping film, spectroscopic plates and others. Cobb and Solomon (49) examined 19 different films and their response to beta radiation from C^{14}. In general it was found that such emulsions were less sensitive than X-ray but had better resolution and less background. Fitzgerald et al. (77) recommended use of medium lantern slide emulsion for histological sections containing moderate concentrations of radioisotope.

The development of special emulsions for autoradiography has paralleled the development of the technique. In this country NTB, NTB-2, NTB-3 and NTA emulsions are available as plates from Eastman Kodak Company. Several of these emulsions may also be obtained in the gel, pellicle or stripping film form. Kodak Limited and Ilford, in England, have also developed excellent nuclear emulsions. Boyd (43) offers a detailed discussion of the available emulsions for this work. In general, the nuclear emulsions are not as sensitive as X-ray emulsions but do have much better resolution and lower background. These emulsions are used when quantitative track or grain counting is the objective.

Resolution in Autoradiography

Resolution in autoradiography is influenced by several factors:

a) Type of radioactive particles emitted.
b) Thickness of the tissue section.
c) Thickness of the emulsion.

d) Distance between the tissue and emulsion.
e) Exposure time.
f) Back scattering of particles from the supporting slide.
g) Grain size and density in the emulsion.

From the theoretical side this phase of autoradiography has been investigated more than any other (58, 139). Stevens (169) experimentally studied resolution by developing radioactive resolution test charts after chemical toning the silver in minute photographic images. Conversion of the silver to silver bromide and then to silver iodide, using I^{131}, was effected with little loss of sharpness. From the known distance between lines the resolution obtainable has been found to be better than 2.5 μ, a close approximation to the theoretical limit discussed by Doniach and Pelc (58).

For more detailed discussion of resolution, reference is made to Boyd (43) and others (14, 26, 59, 77, 86, 92, 98, 99, 100, 139). The most important factor in resolution, as pointed out in all the citations, is the distance between the tissue and emulsion. Theoretical calculations and curves show that if a gap between emulsion and section is reduced from 3 μ to 0.1 μ, the resolving power will be improved from 17 μ to 3 μ (58). Stevens (169) experimentally showed the same effect. With nuclear track emulsion, he reduced the number of lines per millimeter from 220 to 61 by a 3 μ layer of gelatin between the test chart and emulsion.

Determining Radioactive Level in the Tissue and Length of Exposure Time

Boyd (43), in his recent book, gives a detail listing for doses administered to various animals and the conditions of the autoradiographic experiments performed. Wainwright et al. (187) devised a simplified chart for exposure calculations in autoradiography. However, an estimation of the level of radioactive isotope necessary in a plant structure for autoradiography is often impossible to make unless part of the plant material is also used for a radioactive assay. Even under these conditions it is difficult to estimate the fractional part of the plant that might be present in a thin tissue section of a leaf or stem. Easley (64), after exposing plants to $C^{14}O_2$ for 24 hours, removed leaf and stem samples for histological processing. Adjacent sections were dried, ground and

spread on a tacky sample pan for radioactive assay. An attempt to estimate the radioactivity contained in 10 μ section and from this to determine exposure time for a nuclear emulsion was unsuccessful. The most useful way to determine the length of time for exposure is to prepare a series of slides from the same tissue and to process them at intervals of a few days. The interaction of radioisotope level and time of exposure for optimum results is one that will give an autoradiogram sufficient for the information desired.

For physiological or cytological interpretation from autoradiograms, the level of radioisotopes incorporated into plant cells prior to preparation of the tissue should be such that radiation damage does not occur. This is especially true if the period in which the radioisotope is present in the cells prior to fixation is longer than a day. Beal (15) found a marked distortion of the upper epidermis of medicinal plants when they were placed under a bell jar in an atmosphere containing $C^{14}O_2$. The mesophyll of the leaves was also altered by the treatment and in some cases cells were killed. The initial level of $C^{14}O_2$ included 0.9 millicurie (mc) of C^{14} in the 22-liter chamber. This is approximately 2×10^5 disintegrations per minute per cubic milliliter of gas, an extremely high level for the exposure period used.

Russell and Martin (154) added P^{32} to the nutrient solution in which barley plants were grown. They varied the concentration of the radioactive phosphorus from 0 to 50 microcuries (μc) per liter of solution. Significant radiation effect was observed on the roots of the plants when the solution contained 10 μc/l. Pelc and Howard (111, 112, 146, 147), in a series of experiments in which *Vicia faba* plants were grown for 24 hours in radioactive phosphorus solution prior to making autoradiograms for cytological incorporation studies, used solutions containing 0.2 μc/ml. and 16 mg./l. of carrier phosphorus. In other experiments S^{35} at one μc/ml. of solution was used to label root cells for incorporation studies. These investigators computed that the lowest doses in roentgen equivalent physical (REP) to obtain autoradiograms from plants killed 24 hours after the application of the tracer are: for P^{32}, 0.7 with X-ray film and 28 with stripping film; for S^{35} and C^{14}, 0.02 with X-ray film and 0.7 with stripping film. Odebland (140) found histological damage to animal tissue when P^{32} was present at 3 μc/gr. of tissue. Hertz (100) calculated the

number of electrons required per cm.^{2} to obtain a density of 0.6 within 15 days on Eastman no-screen X-ray film. He used the data of MacDonald et al. (131), which showed the activity, in microcuries, necessary to obtain a density of 0.6. The relationship used is:

$$N_e = (\mu c)\ 3.7 \times 10^4\ (1 - e^{-\lambda t})$$

N_e = number of electrons required per cm.^{2}
μc = microcuries
λ = decay constant (sec.$^{-1}$)
t = exposure time in sec.

From this calculation 0.38×10^7 electrons/cm.^{2} from C^{14} are needed to give a density of 0.6. For C^{45}, 2.0×10^7 electrons/cm.^{2} are needed and for P^{32}, 7.2×10^7 electrons/cm.^{2} are needed.

Ehrenberg et al. (65) seed treated barley and wheat seed with various levels of P^{32} and recorded the effect on growth. Five μc/seed caused half of the cell division in the growing plant to have chromosome disturbances. Growth was retarded also. The estimation as to the amount of P^{32} which causes a completely lethal condition, provided the P^{32} acted for ten days, was 18 μc/gr. fresh weight for whole seeds, 40 μc/gr. fresh weight for roots, and 30 μc/gr. fresh weight for green stems. On the other hand Spikes et al. (166) report that the lethal dose of P^{32} and Sr^{90} is approximately 1.4 μc/seed for wheat, barley and sunflower. Autoradiograms made at five weeks growth after seed treatment indicated that P^{32} was fairly uniformly distributed, whereas Sr^{90} accumulated in the first two leaves.

Good autoradiograms were made from anther smears after the inflorescence of *Tradescantia paludosa* and *Lilium longiflorum* had absorbed P^{32} from a solution containing 20 μc/ml. (178). Anthers were fixed eight to 48 hours after absorption started. The author did not mention whether cytological effects caused by the radioisotope were observed.

Ehrenberg and Granhall (66) also injected buds of *Malus*, *Pyrus* and *Prunus* trees with P^{32}. Accumulation in the meristematic tissue was observed. Their estimate of the REP dosage from the P^{32} was believed to be 30 per cent accurate.

At this point in the development of the autoradiographic technique for plant investigation, the best method for determining how

much activity need be present in plant tissue and how long to expose the emulsion is by exposing adjacent sections for increasing periods of time until the desired autoradiogram is obtained. Dugger (61) and Dugger and Moreland (62) obtained autoradiograms from *Coleus* and *Hydrangea* leaf sections that had been exposed to an atmosphere of $C^{14}O_2$. The activity of the leaf material, recorded with a GM tube of five per cent efficiency, was approximately 1,200 c./m./one-inch disk. The autoradiograms were exposed for four weeks. Sections of the material were 15 μ thick and were mounted on NTB-2 emulsion. An approximate calculation from this work would indicate that if a 15 μ section contained enough activity from C^{14} to give 20 counts/minute above background, it would be possible to obtain an autoradiogram with the nuclear emulsions within 30 to 40 days. This time could be reduced if grain, or track counting, were the objective.

METHODS IN AUTORADIOGRAPHY

Preparation of Tissue for Autoradiography

Plant tissue to be used for autoradiography has to be processed prior to placing in contact with the emulsion. For either survey or detail autoradiography, the tissue must be killed and dehydrated. Further processing is necessary if histological sections are to be used, as in detail autoradiography. This involves embedding or preparing the material so that it may be cut into thin sections. Each of these steps as they apply to autoradiography will be considered.

PREPARATION OF PLANT TISSUE FOR GROSS AUTORADIOGRAPHY

The plant or plant parts are usually dried so as to form a flat herbarium type specimen. This has been accomplished, in most cases, by use of a plant press and blotters or a photographic cassette. Pressing the plant under too much pressure will express out the vascular sap which usually contains part of the radioisotope. Drying in a flat position without loss of sap can be done if the plant material is dried between pieces of thin absorbing paper and glass plates. The paper or blotters used in the drying should be replaced before placing the film in contact with the plant. There is the possibility that naturally occurring compounds may

be present in plants that are capable of giving rise to a latent image by chemical action (156, 167). Transfer of the radioisotope within the plant may occur in the drying process (141, 152). This can be prevented, to a degree, by cutting the plant material into small sections before drying; or more completely, by freezing the tissue immedately after harvest and drying while in the frozen state (152). The emulsion and dried plant material may be separated by wax paper, aluminum foil, thin silver foil, plastic films, etc., to prevent chemical fogging; however, if the radioisotope is C^{14}, S^{35} or H^{3}, absorption of the beta rays, by even a thin film, is appreciable. In this case the interposed film may be omitted and an untreated control autoradiogram made to check on possible chemical action.

PREPARATION OF PLANT MATERIAL FOR DETAIL AUTORADIOGRAPHY

In general, preparation of plant material for detail autoradiography involves chemical killing, fixing and dehydration, followed by embedding in paraffin for sectioning with a microtome. Several solutions are available for rapidly fixing plant tissue (118), and several chemical methods have been tried in conjunction with autoradiography (61, 62, 64, 133, 167). McIlrath (133) found that the formalin–acetic acid–alcohol (FAA) method gave excellent histological sections but caused excessive leaching of P^{32} from the tissue. Easley (64) found this to be true with C^{14} incorporated in tobacco plants by photosynthesis. She also showed that carbon leaching from plant tissue may result from either or both of two factors. First, carbon in plant tissue could be fixed better by formalin than by Randolph's fixative. Second, fewer carbon–containing compounds are leached by avoiding the lower alcohol concentrations. Of all the methods used by McIlrath (133), that of the freezing microtome was the only one that yielded satisfactory sections without excessive loss of P^{32}. Easley (64) assayed the tissue and solutions used in dehydration through butyl alcohol and two ethyl alcohol series. She found definite correlation between the amount of radioactivity leached from the tissue and that recovered in the solutions. All three methods leached out more activity than remained in the tissue after the dehydration was completed. Boyd (43) gives Steffey's method (167) used with

Hemerocallis spp. and *Antirrhinum* spp. after absorption of S^{35} through excised flower bearing stems. No check was made on the loss of S^{35} by leaching, however.

Because of the excessive loss of radioactive isotopes from issue by chemical fixation and dehydration, it is necessary to resort to other methods for preparing plant materials for sectioning prior to making autoradiograms. The only other method that has been successful is to dehydrate while the plant is in the frozen state. This method has given excellent results with animal tissue (2, 78, 96, 103, 105–108, 124, 131, 192, 193). The method used by these investigators has been generally adapted from the Altmann technique for freezing-drying. Theory, practice, and application of this method, as well as modifications for special purpose, are given by Bell (21), Gersh (82), Harris et al. (96), Glick (84) and others (83, 85, 135, 160).

Goodspeed and Uber (88, 89) applied the Altmann freezing-drying technique in preparing plant material for cytological studies. They found that plant tissue is much slower to dehydrate in the frozen state than animal tissue. Using anthers of *Lilium longiflorum* and root tips of *Allium cepa*, dehydration under a pressure of 1 μ of Hg was continued 14 days. Jensen (115) found that root material required six days to become dehydrated properly. He was able to effect drying of leaf sections in one to two days. Biddulph (28) dehydrated stem and leaf tissue for three to four days before a pressure of 10^{-4} mm. Hg was recorded. This vacuum was maintained for 24 hours to insure proper drying. For comparison Mendelow and Hamilton (135) obtained good drying of animal tissue in seven hours, and Glick and Malmstrom (85) reported drying in eight hours. Easley (64), using freeze-drying equipment similar in design to the Glick and Malmstrom apparatus, found that with 32 hours of dehydration in the frozen state, good histological sections could be obtained from stem and leaf tissue.

The general method for preparation of plant material for autoradiography by using the freezing-dehydration method is:

a) Small pieces of tissue are removed from the plant and immediately frozen in rapidly stirred isopentane cooled with liquid air or nitrogen.

b) After freezing, the tissue is transferred from the isopentane

directly to the liquid air or nitrogen. The tissue may be left here while other preparations are made.

c) The dehydration chamber which has degassed solidified paraffin in the bottom is cooled to a temperature of −40 to −60° C. A good mixture is diethylcarbitol and dry ice (−54° C.) (189).

d) The tissue is transferred to the chamber and sealed to the vapor-trap and vacuum system.

e) The mechanical pump is started and continued until the pressure gauge indicates a pressure less than 5–10 mm. of Hg.

f) The diffusion pump is then started. This should continue for one day after the pressure gauge indicates 10^{-4} mm. Hg pressure (28, 64).

g) After dehydration the tissue is allowed to slowly warm up to room temperature. Pumping is continued during this warm up stage.

h) The tissue chamber is warmed slowly until the paraffin melts. The tissue will sink in the paraffin.

i) After the paraffin is solidified again, the pumping is stopped and the system is returned to atmospheric pressure.

j) The paraffin and tissue are removed, transferred to an oven and the paraffin changed.

k) The tissue and paraffin are processed as in normal microtechnique method.

Other methods for preparing tissue for autoradiography to prevent loss of isotope by leaching have been described. Blank et al. (29, 30, 31) describe a non-vacuum freezing-dehydration technique for use with animal tissue and bacteria. In this technique the tissue blocks are placed directly into cold propylene glycol stored in a deep freeze (−20° C.). After several hours the tissue is transferred to a mixture of polyethylene glycols at 55° C. for infiltration. The tissues are infiltrated for two to three hours and blocks are prepared with this. Before using the carbowax mixture, it should be heated to 175° C. for 30 seconds to drive off hygroscopic moisture (75). In the cutting of sections, a thin layer of molten paraffin may be added to the block face before each cut (105), and the cut sections pressed directly against the photographic emulsion. These investigators completely reduced leaching loss by this method and showed good autoradiograms. Miles and Linder (136) reviewed the use of polyethylene glycols as histo-

logical embedding media. Attempts to adapt such a method to plant material have proved unsuccessful (60, 64, 133). Holt and Warren (109, 110) had good results by using carbowax with frozen dried animal tissue for autoradiography.

Russell et al. (155) prevented leaching of P^{32} from barley stem tips during preparation by fixing the frozen tissue in isopentane cooled to −170° C. and transferring to absolute alcohol saturated with basic lead acetate at −70° C. Ice in the tissue dissolved slowly in the alcohol and phosphorus precipitated in situ as the lead salt. Silver nitrate has been used to precipitate Br^{82} with no loss from animal tissue infiltrated with paraffin (134).

A quick freeze method for making smear slides permanent may well be adapted to autoradiography (54). Another special method that may be used in combination with plant autoradiography is that of microincineration (183).

Fink (74) mechanically enlarged cells embedded in lead disks by rolling the disk into a thin section. The enlarged tissue containing an alpha emitter was used in autoradiograms. The results were not good.

Preparation of Autoradiograms

TECHNIQUE FOR GROSS AUTORADIOGRAMS

For gross type autoradiography the general method first employed by Arnon et al. (7) to show the distribution of P^{32} in the fruit of the tomato is still used. After the tissue is dried flat, it is placed in contact with an emulsion, usually no-screen X-ray type, with a thin layer of non-absorbing material between the tissue and emulsion, as previously discussed. The approximate level of radioactivity in the tissue can be first determined by a laboratory monitor. If the isotope studied is carbon, sulfur or tritium, an estimate of the activity with such an instrument is not reliable. Fortunately, the half-life of these three isotopes is long enough to allow the investigator to repeat the process with the same specimen if the time of exposure has been under-estimated or over-estimated. Time may be saved if X-ray film is placed on both sides of the dried tissue (60). One film may be processed earlier than the other to see whether longer exposure is necessary. Care must be exercised to keep from moving the position of the tissue relative to the film if such a method is used. The gross type auto-

radiogram is extensively used to show distribution of radioactive elements in plants. It must be born in mind that the autoradiogram is not a picture of the plant part but one of the radioisotope in the plant (173).

In general, this type of autoradiography is not a quantitative method such as grain or track counting. However, it has been used in a semi-quantitative way to compare the distribution of radioactive elements between plants receiving different treatments (27, 151, 164), the comparison being visual. Use of quantitative density measurement has not been adapted to plant autoradiography.

TECHNIQUES FOR DETAIL AUTORADIOGRAMS

Boyd (43), in his book on Autoradiography in Biology and Medicine, devotes an entire section to techniques, and gives in step-wise fashion the various methods that have been developed. Other review articles, previously cited, have also described the methods used in detail autoradiography. These methods, as they have been applied to autoradiography with plant tissue, will be discussed.

a) The apposition method has not been used to any extent with plant tissue. The method involves placing the tissue section on a slide in contact with another glass or plastic-backed emulsion for exposure. The section and emulsion are processed separately after the exposure period, and the two parts can be compared under low magnification or with a binocular scope. Detail localization within cells, however, is not possible with this method. With X-ray film strips, Easley (64) applied the method to determine the approximate level of C^{14} in plant tissue to be used for more detail studies with nuclear emulsions. Because X-ray emulsions are much faster than nuclear emulsions, the time of exposure for the latter could be estimated prior to development.

b) Mounting of thin tissue sections onto the photographic emulsion has been used with plant materials more than other techniques. This is due in part to the availability of the nuclear emulsions prepared in this way. Endicott and Yagoda (68) and Evans (69, 70, 71) first used this method with animal tissue. The paraffin ribbons of tissue sections were floated on warm water and then transferred to cool water to harden the ribbon. In the dark

room with the safe lamp on, the ribbons were removed from the water with a section lifter and placed on the emulsions previously swollen by soaking in distilled water. This method has been used in plant studies with fair to good results (61, 62, 64, 171); however, loss of soluble radioisotope from the sections will often occur during the preparation. Leaching into the floating solution or the swollen emulsion may result in artifacts that affect the usefulness of the autoradiograms.

Attempts to prevent leaching from the tissue while placing the sections on the emulsion have been made. Holt et al. (105) used liquid paraffin to adhere the section to the emulsion. Harris et al. (96) floated sections onto clean mercury at 50° C. This temperature softened the paraffin, and in such a state the ribbons were pressed on the slide or emulsion. Gibbs, according to Boyd (43), used 95 per cent alcohol for floating the sections onto the slides without leaching. Several methods for dry mounting paraffin sections without the use of an adhesive or floating solution have been used (81, 102, 190).

Excellent results have been obtained with this technique. Boyd and Levi (41) employed it for track autoradiography, and Boyd et al. (37, 42) also relied on this method for grain counting. Both uses of the method are quantitative. This technique has also served to obtain high resolution autoradiograms from radioisotope in paramecia and yeast (67, 76, 162). Tritium-labeled cells were cut at 1 μ prier to floating onto the emulsion. Campbell (46, 47) also used this method for quantitative track counting with autoradiograms of isotopes incorporated into rat tissue.

c) The use of liquid emulsion to coat histological sections mounted on glass slides was developed by Bélanger and Lebland (18). Medium lantern slides were soaked in distilled water at room temperature. The swollen emulsion was scraped off into a beaker and melted at 35° to 40° C. In the darkroom the liquid emulsion was pipetted onto the slide and spread with a camel hair brush. After exposure and development of the emulsion the tissue sections were stained. Modificatoins and improvements on this method have been made by several investigators (6, 20, 33, 79, 93, 119, 125, 126, 128, 148). Modifications have also been used with plant tissue sections (28, 64, 116, 167).

d) Pelc (144) prepared autoradiograms by superimposing an

emulsion, stripped from the backing material, onto thin sections. Boyd and Williams (38) and MacDonald et al. (130) also were early users of the "stripping film technique". The first work with this technique was done with emulsions developed for other work. Kodak autoradiographic plates and Permeable-Base stripping film were later developed with a gelatin layer about 10 μ thick as a support. The emulsion is placed in contact with the tissue section, and after exposure is processed through the gelatin backing. These emulsions are 4–5 μ thick when prepared, and dry down in the final autoradiogram to approximately 2 μ. Excellent results have been obtained with this method; Pelc and co-workers (59, 111, 112, 145, 146, 147) applied it in their investigation of P^{32} and S^{35} incorporation into the protoplasm and nucleus of *Vicia faba*. Other investigators have also used the technique (48, 64, 67, 123, 129, 155, 176, 177, 178, 192).

e) Bélanger (19) modified the coating technique previously discussed by removing the developed emulsion, with tissue attached, from the slide, inverting the emulsion onto another slide and staining the tissue section. The big advantage of this method is that the tissue sections do not come in contact with the developer, nor does the emulsion come in contact with the histological stain. Levi (127) used this inverted technique for track counting.

f) Gomberg (86) developed a new high-resolution system of autoradiography called the "wet-process method". In this process a photographic film is synthesized on the slide containing the tissue section, and the photographic development is physical by a ferrous sulfate solution. Gomberg used this method to get one μ resolution with C^{14} autoradiograms. There are several disadvantages to the method, however. The entire process is limited in application, as it must be completed in less than a week. Easley (64) tried the method with plant material without much success. Other methods for preparation of emulsion to use in autoradiography have been attempted (163).

g) Autoradiograms have been made to locate the site of boron in cellular materials. Mayr et al. (132) fed rats B^{-10}-enriched H_3BO_3. The tissue prepared from these rats was placed on NTA nuclear film, and the entire slide put in a neutron flux of 5.76×10^3 neutrons/cm.^{2}/sec. for ten days. On capture of a neutron by the B^{10} nucleus ($B^{10} + N^1 \rightarrow B^{11}$), the B^{11} nucleus (with

a half life of 0.02 sec.) emits alpha particles of 2.316 and 2.794 Mev-energy. These energetic alpha particles will cause a developable latent image to be formed in the emulsion. This method has also been used to study boron in steel (101). It is possible to increase the sensitivity of alpha particle detection by autoradiography with a thin layer of ZnS-Ag phosphor between the film and source (174).

AUTORADIOGRAPHY IN PAPER CHROMATOGRAPHY

The use of X-ray films for making autoradiograms of chromatographically separated labeled materials was first introduced by Fink et al. (72, 73). The technique was used by Calvin and coworkers (22, 23, 44, 45) in studying the path of carbon in photosynthesis and the identity of compounds synthesized by *Chlorella* cells from $C^{14}O_2$. Many other reports have appeared in the literature in which autoradiography has been used in conjunction with chromatography. Block et al. (32) briefly reviews the technique in his recent book on Paper Chromatography and Paper Electrophoresis. Aronoff (11) also discusses this application in his book Techniques in Radiobiochemistry. Autoradiograms of this type have been used to help determine the purity of biosynthesized compounds (8, 9, 149, 161, 184, 185).

PHOTOGRAPHIC PROCESSING

Processing of autoradiograms after exposure is a very important part of autoradiography. It is in this phase of the work that many of the artifacts in autoradiograms are produced. Boyd (43), in the section on techniques in Autoradiography in Biology and Medicine, gives detailed methods for processing the various kinds of autoradiograms. Individual methods reported in the literature previously cited usually include a section on the photographic process employed in the technique. Some of the important considerations that have been emphasized by workers are:

a) The photographic solutions used in processing the emulsion should be free of trash and dust. Suggestions have been made to use freshly distilled water and chemically pure compounds. The solutions should be filtered before using.

b) All light except the safe light recommended by the film manufacturer should be excluded.

c) The temperature of the developing solutions should be controlled according to the recommended value. Variations in the temperature in the period of time needed to process several autoradiograms will cause noticeable changes in the end results.

d) The developing period determines the size of the individual silver grains. The objective of the particular process will dictate the desirable grain size. For quantitative autoradiography small grains are essential. Some workers in the field recommend overexposure and under-development for good autoradiograms with low background.

e) Fixation should be twice as long as it takes to completely clear the film. The time for fixing is usually recommended by the emulsion manufacturer. An acid fixative is recommended to harden the emulsion.

f) Use of agitation in the photographic process may cause separation of the emulsion and tissue section. The possibility of this happening depends on the kind of autoradiogram made. If sections are mounted on slides of nuclear emulsion, agitation will sometimes cause loss of the sections. In those techniques in which the emulsion is sealed over the section, there is less likelihood of separation. Agitation should be done very gently.

g) Washing the autoradiograms in running water for 30 to 60 minutes after fixation is recommended. Filtered distilled water should be used if available.

h) Drying the autoradiogram may be done chemically if staining is to follow. Usually drying in a gentle stream of cool air is recommended.

ARTIFACTS IN AUTORADIOGRAPHY

Formation of a latent image in the emulsion from means other than the action of the radioactive isotope incorporated into the biological material often occurs. This may come about for one of several reasons.

a) In placing the emulsion and histological section in contact with each other, the manipulation of the two might cause artifacts from:

(1) fingerprints on the emulsion.
(2) pressure on the emulsion.

(3) static electricity caused by stripping the emulsion from the glass or plastic backing.
(4) holding the emulsion too close to the safe light.

b) The action of components in the biological material often brings about the formation of artifacts. Artifacts of this type have been previously noted. Russel (156, 157) found that vegetable materials darken photographic plates. The principal constituent of resins that caused this was abietic acid. Several observers (43) have noticed that plant material will cause formation of a latent image without having a radioactive isotope present. Similar images from the leaves of tobacco plants dried and placed in contact with X-ray film have been observed (60). Aggregation of developed silver grains in autoradiograms of plant material also have been observed (64, 167). Chemical compounds present in biological tissue, such as cysteine, glutathione and dimercaptopropylurea, were effective in latent image formation (34, 39). The authors thought this might be caused by the actions of compound containing R-SH groups. Other chemicals have been reported to cause fogging of photographic emulsions (43, 197). Chemicals present in the tissue also have been noted to prevent the development of latent image (43). Easley (64) had a clearing of the emulsion formed in "the wet process method" around the plant tissue sections. Background fog occurred where the emulsion was not over the sections.

Odebland (140) mentioned that about one-half of the autoradiograms in his work had minor or major artifacts. The significance of artifacts is therefore evident, and their high incident is primarily related to the many possibilities for obtaining them. He says that "the greatest care must be taken therefore, in autoradiographic practice, not only if quantitative autoradiography be employed but also for qualitative conclusions".

APPLICATION OF AUTORADIOGRAPHY IN PLANT STUDIES

Physiological Application in Higher Plants

Phosphorus 32 has been the radioisotope most widely used in autoradiographic studies with plants. This has been related to: *a*) accumulation of phosphorus by plants to a concentration greater than exists in the soil or nutrient solution, *b*) availability and low cost of P^{32}, *c*) high energy associated with the beta particle when

P^{32} decays, *d*) half-life. Accumulation of P^{32} in the young leaves and stem tips of tomato plants was shown by Arnon, Stout and Sipos (7) in 1940. They concluded from gross autoradiograms that young tomato fruit accumulated more P^{32} than older fruit, and that phosphorus in the leaves of tomato was re-transported to fruits when the phosphorus content of the nutrient solution became low. Hamilton (95) cited a personal communication from Stout and showed a gross autoradiogram of P^{32} transport out of tomato leaves when the labeled Na_2HPO_4 was applied to the leaf. Later work with gross autoradiograms showed that tomato, bean and corn leaves are efficient organs for absorbing phosphorus (194).

Colwell (51) scalded the petiole of a squash plant after putting one μc of P^{32} as Na_2HPO_4 in a plasticine ring sealed to the leaf. Under this condition, transport of phosphorus out of the treated leaf did not occur. Without scalding, the cross sections of the stem in contact with no-screen X-ray film produced an autoradiogram from the P^{32} in the phloem. Klechkobsky and Evdokimova (120) correlated gross autoradiographic information with total activity in the leaves, roots, stems and fruits of corn. With P^{32} it appeared from the autoradiograms that there was more isotope located in the leaf veins than in the interveinal tissue, but on analysis they found about three times the activity in the interveinal tissue as in the veinal tissue. With S^{35} the gross autoradiograms showed that the interveinal tissue contained most of the isotope. The apparent lower value for S^{35} in the veins, as interpreted from the autoradiograms, was caused by the more complete absorption of the weak beta rays by the larger mass of veinal tissue. They further point out that autoradiograms from radioisotopes in plant material depends on: *a*) the energy of the isotope, *b*) density of tissue above the emulsions, *c*) amount of radioisotope absorbed.

Colwell (52) labeled pine and Douglas-fir pollen with P^{32} by vacuum infiltration and collected the pollen on canvas filters after it had been disseminated by wind. The canvas filter and collected pollen were used to make autoradiograms. The pattern of distribution was thus determined.

The gross autoradiographic technique has been used to study the influence of aluminum in the culture solution on the distribution of phosphorus in barley plants (195). When aluminum was present in the solution, P^{32} was shown to accumulate in the root

systems with very little movement to the tops. Without aluminum the autoradiograms showed that P^{32} was transported to the tops of the barley plants. In a later study in the same laboratory, it was confirmed by gross autoradiography that the rate of transport of P^{32} to the tops of sunflower plants was dependent on the transpiration rate (196).

Russel et al. (155) used the detail autoradiographic technique to show the distribution of P^{32} in barley plants. The stripping film technique was used to show an accumulation of P^{32} in leaf primordia, base of younger leaf sheaths and initial of the adventitious roots.

The transfer of *Sequoia* callus tissue to media containing radioisotopes and the subsequent accumulation by the tissue has been studied by autoradiography (13). The culture medium contained one to one and one-half millicurie of radioisotopes per liter at the beginning of the transfer. Autoradiograms showed an accumulation of the isotopes in the marginal meristem, the cambia around the tracheid groups, the callus cells exposed to the air, and in the absorptive cells that were in contact with the nutrient agar. P^{32} accumulated in this tissue more than S^{35} and C^{14} from sucrose-C^{14}. The author mentioned leaching during the preparation stage but did not attempt to measure it. Biddulph (28) injected P^{32} and S^{35} into bean plants by the leaf-flap method. The autoradiograms were prepared from sections 15 μ thick and with Ansco liquid emulsion A. She showed that most of the activity was in the phloem tissue, but not all the phloem in any one area of the stem functions as a unit of translocation. Autoradiograms made from the roots of pine trees after periods in which P^{32} was absorbed showed that the portions of pine roots with mycorrhizal fungi accumulated larger quantities of P^{32} than the portions that are non-mycorrhizal (121). The authors attributed this to greater surface and higher respiration in the mycorrhizal roots. The maximum absorption of P^{32} was not in the root tips but in older regions where mycorrhizal branch roots developed. Later extension of this work (122) showed autoradiograms of tomato, barley, and pine roots that correlated the activity present with analytical data obtained by radioactive assay.

Harrison, Thomas and Hill (97) prepared autoradiograms from sections of wheat plants cut on a freezing microtome after embed-

ding them in paraffin following butyl alcohol dehydration. The wheat plants had been labeled with S^{35} by absorbing labeled sulfate from the nutrient solution or from absorbing labeled sulfur dioxide gas. In the sections cut by the freezing method alone, drying and shrinkage occurred so that it was impossible to correlate film density and the corresponding structure of the plant section. They found that the distribution of S^{35} in the wheat plant was similar to the distribution of the protein. There was little, if any, difference in the distribution between the two methods of labeling. In a later report (179) the autoradiographic technique was used to show the accumulation of S^{35} and As^{76} in sugar beet, tomato, radish and bean. It was found that translocation of S^{35} absorbed as $S^{35}O_2$ by the leaves is slower than translocation of S^{35} absorbed as $S^{35}O_4$ through the roots. The absorption of $S^{35}O_2$ by beet leaves appeared unevenly as shown by autoradiograms. Arsenic^{76} appeared in both xylem and phloem of beet after 21 hours of absorption, whereas in bean leaves, labeled by the leaf dip technique, very little radioactive arsenic moved out of the leaves.

The absorption of $S^{35}O_2$ by alfalfa plants (80) and of elemental S^{35} by lemon peel (181, 182) has been studied with the aid of autoradiograms. In the latter study the lemon peel became weakly labeled by the treatment. Gross autoradiograms of plants containing S^{35} to show absorption and translocation of sulfur have also been used by Hofmann and Suss (104) and by Biddulph et al. (27).

A comparison in the availability of Ca^{45} in various chemical forms to alfalfa, tomato, red clover and wheat plants has been made by autoradiography (153). The investigators found that the carbonate form was more available than the biphosphate or sulfate form. With the split root technique, they further showed by autoradiograms that Ca^{45} was not transferred from one part of the root to another.

Gross autoradiograms of plants containing radioactive minor elements in plant nutrition have been made by several investigators. Stout et al. (172) grew tomato plants to fruiting stage with Zn^{65} as the sole source of zinc in the nutrient solution. From the autoradiograms the authors concluded that Zn^{65} accumulated in the seeds and conductive tissues of the fruit more than in the pulp, and that the zone of abscission of the fruit peduncle absorbed more

Zn^{65} than the tissue above or below it. Quantitative analysis of the fruit parts substantiated this. In the same laboratory, short time absorption studies of Mo^{93} and Mo^{99} by tomato plants were conducted (173). The autoradiograms showed a distinctly different type of distribution of molybdenum in plants than with other mineral elements. Accumulation occurred in the interveinal area but not in the stem, and a large number of stomates accumulated the element. The influence of phosphate on molybdenum translocation was also shown by gross autoradiograms.

Rediske and Biddulph (151) used the gross autoradiographic method in a semi-quantitative manner to show the interrelations of hydrogen ion concentration and phosphate content of the nutrient solution on the absorption and translocation of Fe^{55} in bean plants. They found that the absorption and redistribution of iron within plants is at a minimum at high iron concentration, high phosphate concentration and high pH. Gross autoradiograms of radioactive iron in citrus leaves have been used to show the difference in the distribution of iron supplied as the Fe^{55}EDTA and as the $Fe^{55}SO_4$ (170). The radioactive iron as the Fe^{55}EDTA was more evenly distributed throughout the leaves than the iron supplied by $Fe^{55}SO_4$.

Autoradiograms of Fe^{55} in oat plants have been used to study the relationship between nickel-toxicity symptoms and the absorption of iron (57). They showed that the chlorotic areas in the leaves match areas in autoradiograms having a lower content of Fe^{55} than healthy tissue. Toxic levels of nickel in the soil caused a necrosis in the plants with an associated lower concentration of iron in the necrotic areas.

Millikan (137) made gross type autoradiograms of flax, pea, cabbage and tomato plants growing in nutrient solutions to which Mn^{54} was added. The distribution of Mn^{54} in the plants, as shown by radioactive assay and autoradiogram, was a function of the applied molybdenum. In general, the Mn^{54} was more uniformly distributed throughout the plants, and the level was lower when molybdenum was added. Under toxic levels of manganese, necrotic areas in the leaves of plants were associated with a marked accumulation of Mn^{54} in the necrotic tissue. The effect of silicon on yield, Mn^{54}-uptake and distribution in the leaves of barley plants grown in culture solutions, has recently been studied (191).

Autoradiograms of barley leaves, showing manganese toxicity symptoms on plants grown without silicon, showed distinct islands of radioactivity which coincided with necrotic spots in the leaves. With silicon in the nutrient solution, there were no visible symptoms of toxicity, and the autoradiograms showed no radioactive islands. Silicon seemed to disperse or chelate manganese.

Autoradiography has also been used to study the absorption and distribution of fission produced in barley and corn (114, 158, 159).

Radioactive carbon introduced into plants as organic compounds or as $C^{14}O_2$ and used in physiological studies after incorporation by photosynthesis is an excellent isotope to study by certain autoradiographic techniques. On the other hand, the soft radiation may be completely absorbed by an interposing layer of material between a dried leaf and an X-ray film. In general, C^{14} has been used more extensively in detail autoradiography than in gross autoradiography. Detail and gross autoradiograms, in conjunction with counting data, has served to show the permeability of non-stomate epidermis of leaves, fruits and roots to $C^{14}O_2$ (60, 61). Later work (62) showed that the palisade tissue in *Hydrangea* and *Coleus* incorporated much more C^{14} from $C^{14}O_2$ than the spongy tissue. Rabideau and Mericle (150) exposed corn shoots to $C^{14}O_2$ and made autoradiograms of the root apices after paraffin embedding. They found the greatest radioactivity in the terminal millimeter of roots, with the inner cortex, outer stele and superficial cells containing the most C^{14}.

Autoradiograms were used by Earle et al. (63) to show the translocation of C^{14}-labeled 2,4-dichlorophenoxyacetic acid. They found no marked differences in absorption by plants exposed for short intervals as compared with those exposed for longer intervals. Equilibrium in absorption was established at 24 hours with maximum distribution at eight hours. Young bean plants previously treated with a small quantity of labeled 2,4-dichlorophenoxyacetic acid have been used to make autoradiograms (56, 141, 152); the transport of the growth regulator and factors influencing the transport were studied. Tryptophan C^{14} was fed to barley leaves, and autoradiograms were made to show regions of accumulation (36). By using the isotope dilution method, the investigators were able to show that the activity observed at the tips of the leaves was 58 per cent gramine and in the lower part of the leaves

the activity was 77 per cent tryptophan or the precursor. Aronoff (10) studied translocation of C^{14}-labeled photosynthate in soybean plants. The gross autoradiograms presented showed rapid translocation out of the leaf area exposed to the $C^{14}O_2$. Translocation to adjacent leaves does not occur, even if the level of carbohydrate has been reduced by exposure of the adjacent leaf to darkness. Autoradiograms of C^{14} in young tomato plants, sufficient and deficient in boron, after a four-hour period of photosynthesis in the presence of $C^{14}O_2$ showed that the translocation of the C^{14} photosynthate in the boron-deficient plants was greatly retarded (164). Analytical data corroborated the results shown by the autoradiograms.

Application in Microorganisms

Autoradiograms of isotopes incorporated into microorganisms have, in general, shown good detail. Preparation of these materials for autoradiography is more rapid, and the resolution of the results is usually much better.

Nitella and *Chara* cells were labeled by placing the tip cells in 0.001 M sodium phosphate solution containing P^{32} (138). The algae cells were dried by freeze dehydration and placed in contact with X-ray film. Movement of P^{32} was evident from the autoradiograms, and accumulation occurred at the nodes of the algae cells. Coffin et al. (50) put 100 mc of P^{32} into a small lake for further ecological and physiological studies with the animals and plants inhabiting the water. Autoradiograms of the species in the lake showed that there was more accumulation of the isotope in frogs and fish than in the plants growing from the bottom of the lake. Autoradiograms of C^{14} in amoeba cells, fed with ciliates labeled with C^{14} *Chlorella* cells, showed that the C^{14} was confined to the food vacuoles in the early stages after feeding. Later autoradiograms showed uniform distribution of the C^{14} (5).

Andresen (4) made detail autoradiograms of approximately one micron resolution with *Scenedesmus* cells labeled with tritiated water. After drying the cells onto a slide, Kodak autoradiographic stripping emulsion was placed over the cells. Detailed autoradiograms sufficient for grain counting have been made with *Scenedesmus* and yeast cells labeled with P^{32}, S^{35} or C^{14} (128). The cells

were mixed with liquid nuclear emulsion and poured onto slides so as to have emulsion surrounding the cells. Eidinoff et al. (67, 76) also got excellent autoradiograms from H^3-labeled yeast and paramecia cells with the stripping film technique. Canny (48) obtained high resolution autoradiograms from *Chlorella* cells labeled with radioactive strontium.

Fungi containing radioisotopes have been prepared for autoradiograms. Pearson et al. (142) labeled *Fusarium* cultures and later placed the cultured mycelium in contact with lantern slide emulsion to obtain gross autoradiograms. These same investigators (143) later labeled pathogenic and non-pathogenic fungi with I^{131} and administered the organisms intravenously into animal tissues. The distribution of the fungi in the animal was followed by the autoradiographic technique. Wheeler (188) tagged several kinds of plant pathogenic fungi with C^{14} and subsequently made autoradiograms of the labeled organism on nuclear track emulsion. Adams and Miller (1) labeled *Aspergillus, Penicillium* and *Hormodendrum* with P^{32} and showed autoradiograms of the distribution of the radioisotope in the mycelium and conidia. Selective absorption of S^{35} by the urodinial stage of rust on bean and sunflower leaves, after exposing the infected leaves to sulfur vapors, showed that the infected areas of the leaves accumulated more S^{35}, as seen from the gross autoradiograms, than the uninfected areas (198).

Stonier (171) obtained autoradiographic evidence for the intercellular location of crown gall bacteria by labeling the bacteria with P^{32} prior to infecting the sunflower tissue. Good detail autoradiograms from the emitted beta particles showed that the bacteria were located outside the pith cells.

Cytological Studies with Autoradiography

In conjunction with studies on the lethal effects of absorbed radioisotopes by plants, Spinks et al. (166) showed by autoradiograms that in five-week-old wheat plants, strontium is unevenly distributed, with practically all if it being concentrated in the first two leaves. Phosphorus was fairly uniformly distributed. Ehrenberg and co-workers, in a series of papers (65, 66, 94), also relied on the autoradiographic techniques in their studies of the effects of

electrons, positrons and alpha particles on plant development, seedling lethality and chromosome distribution in wheat, barley, *Allium, Malus, Pyrus* and *Prunus.*

Howard and Pelc (111, 112, 113, 146, 147) have extensively investigated chromosome metabolism in *Vicia faba* by techniques of autoradiography. They have shown that P^{32} is organically bound into desoxyribonucleic acid (DNA) in the nucleus during the resting stage, but not during actual division. The incorporation of S^{35} into compounds within the cell occurred at approximately the same time as DNA synthesis. This S^{35} is found in the chromosomes, distributed along them in a constant pattern. X-radiation (13×10^3 ergs/gm.) of the bean roots reduced the number of cells which synthesized DNA in 24 hours to about one-third of the controls. This same X-ray dosage had no apparent effect on the uptake of S^{35} during the same period. Taylor and Taylor (178) extended the techniques of Howard and Pelc to a cytogenetic study of the distribution of P^{32} and S^{35} in cells of *Lilium* and *Tradescantia* anthers.

Incorporation of C^{14}-labeled adenine and phenylalanine by onion root cells, as demonstrated by autoradiograms, was shown by Jensen (116). The results indicate that the rate of nucleic acid or protein incorporated at any one time depends on the morphological stage of the root cells. There were marked differences in the rate of incorporation between the tissues of the root. The differentiating vascular tissue shows a significantly higher rate of incorporation of adenine than the other tissues.

SUMMARY

Autoradiography, a technique combining radiochemistry, microtechnique and photography, offers another method for studying the morphology, physiology and cytology of plants. As applied to higher plants, the technique has not been thoroughly investigated; however, autoradiograms have been made that show not only good cellular detail but also developed grains of high photographic resolution associated with the cell components.

The techniques of autoradiography in plants should be useful in elucidating such problems as organic transport, inorganic absorption and the pathway involved in absorption, cytological incorporation and synthesis in plants.

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ADDENDUM

In an article (175a) published since the present one was completed, the conclusion drawn by the authors, as evident from the very excellent autoradiograms, is that DNA is synthesized in the chromosome as a unit extending the length of chromosome.

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INTERRELATIONS OF DISSOLVED ORGANIC MATTER AND PHYTOPLANKTON

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INTRODUCTION

Lucas (74–77) has reviewed the possible effects of organic metabolites from an evolutionary and an ecological point of view. He has drawn information from diverse spheres of biology to indicate that organic matter may act upon living organisms directly and indirectly, and affect the dynamics of integration within an ecosystem. It is well known that cells may liberate certain metabolites which influence other cells within or without the organism. These metabolites are evident in the form of hormones, growth stimulators, toxins, antibiotics, embryological organizers, agents of chemotaxis, and many other agents.

The algae occupy the most basic level in the transfer of energy through natural aquatic systems. The distribution and occurrence of algae in both time and space are related to certain fundamental factors which may in general be divided into three categories: physical, chemical, biological. The chemical and biological factors are not so readily separated from each other as from the physical factors. These factors may be listed and subdivided in general as indicated in Table I.

The activity of organic matter falls under both chemical and biological factors. It comprises only a small part of the total number of factors; nevertheless, its effects may be very significant when integrated to the whole. Two major questions may be asked regarding the role of organic matter. From where may the dissolved organic matter be derived and what is the evidence for its presence in natural bodies of water? What may be its effects on living organisms and what is the evidence for these effects?

Dissolved organic matter may include a certain amount of organic debris, some colloidal substance, and truly dissolved organic matter. It is the truly dissolved organic matter that is of primary interest here. Its presence in natural bodies of water has been definitely established (18, 19, 39, 131).

TABLE I

FACTORS AFFECTING DISTRIBUTION AND OCCURRENCE OF ALGAE

PHYSICAL	
Water movement	(44, 50, 52, 64)
Light	
quality	(52, 83)
intensity	(52, 53, 55, 64, 83, 85, 106, 110)
duration	(50, 52, 53, 83, 110)
Temperature	(21, 52, 53, 55, 64, 83, 106, 110)
CHEMICAL	
Nutrients	
inorganic	(21, 44, 50, 52, 53, 55, 64, 83, 85, 92, 106, 110)
organic	(21, 44, 53, 55, 64, 85, 92, 103)
trace elements	(52, 85, 103, 110)
Hydrogen ion concentration	(21, 53, 64, 83, 92)
BIOLOGICAL	
Acclimation	(21, 55, 76, 83)
Accessory growth factors	(21, 53, 55, 64, 75, 76, 85, 103)
Autotoxins	(21, 75, 76, 83, 85, 103)
Antibiotics	(21, 75, 76, 83, 85, 103)
Predation	(50, 52, 53, 64, 75, 106)

What are the sources of dissolved organic matter in natural waters? Organic matter may be derived from runoff of the drainage area, autolysis of organisms in the system, excretion and secretion of living organisms, defecation, and from sediments through erosion or diffusional processes. In addition, existing organic substances may be transformed to new organic compounds by microorganisms or their free enzymes.

EVIDENCE FOR THE PRESENCE OF ORGANIC MATTER

What is the evidence for the presence of dissolved organic matter in natural waters? In order to culture many algae in the laboratory, extracts from soil, soil-water, lake water, yeast or peptone are necessary in addition to synthetic media (21, 24, 85, 101–103, 110). This indicates that trace metals or organic growth factors are required by these algae in order to grow and reproduce. If the algae require organic growth factors in order to be cultured in the laboratory, it seems not unreasonable to expect that they would have the same requirements in natural systems. Thus there is indirect evidence for the occurrence of dissolved organic matter. The evaporation of water samples and ignition of the residue indicates the presence of organic matter. Total dissolved solids, in general, in fresh waters range from about 15 to 300 ppm. Total

dissolved organic matter for Wisconsin lakes ranges from 2.9 to 39.6 ppm. with an average of 12.8 ppm. (131). Fox et al. (39) have found that 40 per cent of the total dispersed organic matter in sea water is dissolved. Peptides, amide nitrogen, glutamic acid, alanine, valine, leucine, glutamine, glycine or serine, pentose sugar, aspartic acid and a complex polysaccharide were demonstrated to be present in the filtrate from algal cultures (20, 35, 38, 128). One would expect these organic compounds to be present also in natural waters. Aleyev (4) has found indications that autolysis of algal cells plays an important part in the accumulation of organic substances in the surroundings inhabited by algae. Johnston (63) has evidence for biologically active organic compounds in the sea. Lake Glubokoje contains 96 ppm. of organic matter. The bulk of this is high molecular weight fatty acids and colloidal complexes (43). Substances which reduce Fehling's solution are also present. Direct evidence has also been obtained by chemical and biochemical analyses of natural waters and extracts from lake sediments. Thiamine, niacin, biotin, three chlorophyll-like pigments, α- and β-carotene, lycopene, myxoxanthin, rhodovialascin, peptides, tryptophane, tyrosine, histidine, cystine, arginine, glucose, galactose, arabinose, xylose, ribose, dehydroascorbic acid and a rhamnoside have been demonstrated (28, 54, 56, 57, 93, 123, 125).

FUNCTIONS OF DISSOLVED ORGANIC MATTER

Dissolved organic matter may exert its effects on organisms in four basic ways: *a*) it may supply compounds utilized as energy sources or which contain the basic elements essential to the building of protoplasm, primarily a problem of nutrition; *b*) it may supply accessory growth factors which are required for or merely stimulate the growth of organisms; *c*) it may be in the form of toxic substances which inhibit the activities or cause the death of many aquatic organisms; *d*) it may form organic complexes with trace metals, the effect of which may be beneficial or detrimental, depending upon the circumstances.

NUTRIENT FUNCTION

It is known that certain algae which are capable of a completely autotrophic existence nevertheless are able to utilize organic substrates in the light as sole sources of basic elements (36). The

experiments confirming this have been conducted with pure cultures in the laboratory. It should be mentioned that Krogh et al. (65), from culture experiments, believed that the organic material synthesized by algae is almost quantitatively stored in the cells of the algae, while a fraction amounting at most to ten per cent may possibly be lost to the surrounding water. They believe that the organic substances lost during assimilation are wholly negligible but that this does not preclude a significance to organic matter derived from other processes. Algeus (5–12) has demonstrated that 13 species of autotrophic algae of the genera *Scenedesmus, Chlorella, Haematococcus, Ankistrodesmus, Stichococcus, Hormidium* and *Zygnema* are able to utilize alanine, aspartic acid, asparagine, glutamic acid, glycine and succinamide to varying degrees as sole sources of nitrogen.

Table II lists algae which have been demonstrated as capable of utilizing organic substrates for energy or growth (104).

TABLE II

ALGAE CAPABLE OF UTILIZING ORGANIC SUBSTANCES FOR ENERGY OR GROWTH

Chlamydomonas		*Cystococcus*	
agloeformis	(46, 80)	sp.	(22, 23)
dorsoventralis	(77)	*Euglena*	
monoica	(77)	*gracilis*	(31, 58, 85, 98, 99)
pseudococcum	(77)	*gracilis* var.	
pseudogloe	(77)	*bacillaris*	(46, 58, 79)
pulchra	(77)	*stellata*	(46)
reinhardi	(113)	*Mannochloris*	
subglobosa	(77)	*bacillaris*	(15)
Chlorella		*Navicula*	
ellipsoidea	(126)	*minima*	(69)
pyrenoidosa	(40, 41, 42, 87, 123)	*pelliculosa*	(69)
		Nostoc	
variegata	(15)	*punctiforme*	(47)
viscosa	(15)	*Polytoma*	
vulgaris	(15, 17, 32, 84, 87 88)	*uvella*	(100)
		Scenedesmus	
Chlorococcum		*acutus*	(26)
humicola	(15)	*basiliensis*	(40)
Chlorogonium		*costulatus* var.	
elongatum	(15)	*chlorelloides*	(22, 23)
euchlorium	(46, 78, 97, 98)	*quadricauda*	(122)
Coelastrum		*Stichococcus*	
Proboscideum	(4)	*bacillaris*	(40)
Cylindrospermum			
sp.	(129)		

Table III lists the organic substrates utilized by one or several of the algae in Table II (104).

TABLE III

ORGANIC SUBSTRATES UTILIZED BY ALGAE FOR ENERGY OR GROWTH

CARBOHYDRATE			
arabinose	(15)	mannitol	(127)
cellobiose	(89)	mannose	(22, 42, 78, 122)
erythritol	(127)	melezitose	(15)
fructose	(69, 78, 89, 122, 127, 22)	methyl-B-D-glucoside (aesculin)	(89)
galactose	(15, 22, 42, 47, 78, 89, 122, 127)	raffinose	(15, 127)
glucose	(15, 17, 22, 23, 26, 32, 41, 42, 47, 69, 78, 84, 88, 89, 122, 127, 129)	rhamnose	(15)
		sucrose	(15, 17, 22, 23, 78, 127)
		trehalose	(15)
inositol	(15)	xylose	(15, 127)
lactose	(15, 22, 47, 89)	dextrin	(47)
levulose	(15)	inulin	(15, 47)
maltose	(15, 17, 22)	starch	(15, 17, 47)
ALDEHYDES AND KETONES			
glyceraldehyde	(98)	valeraldehyde	(40, 42)
acetaldehyde	(40, 42)	dioxyacetone	(98, 100)
propylaldehyde	(40)		
ALCOHOL			
ethanol	(58)	glycerol	(22, 69)
ESTER			
ethyl-acetate	(98)		
FATTY ACIDS			
acetate	(32, 40, 42, 46, 58, 78, 79, 80, 81, 85, 86, 98, 99, 100, 114, 127)	capronic	(97, 127)
		caprylic	(127)
		nonylic	(127)
		undecylic	(127)
proprionate	(32, 97, 127)	oenanthic	(127)
butyrate	(32, 40, 42, 46, 58, 79, 85, 98, 100, 127)	lauric	(127)
		myristic	(127)
		palmitic	(127)
caproate	(79)	stearic	(127)
valerate	(40, 42, 98, 127)		
ORGANIC ACIDS			
aconitate	(32)	malate	(32, 47, 98)
citrate	(47)	pyroracemic	(127)
fumarate	(32)	pyruvate	(32, 80, 98)
α-ketoglutarate	(32)	succinate	(32, 46, 58, 98, 99, 100)
lactate	(32, 46, 98, 100, 127)		
AMINO ACIDS AND RELATED COMPOUNDS			
alanine	(15, 40, 42, 46, 58)	leucine	(15, 40, 42, 46, 127)
asparagine	(40, 42, 46, 78, 99)	peptone	(15, 26, 47, 78, 79, 81, 84, 99)
aspartate	(15, 127)		
arginine	(15)	phenylalanine	(15, 46)
cystine	(15, 127)	serine	(15, 99)
gelatine	(15, 17, 78)	tryptophane	(15, 127)
glutamate	(15, 58, 99)	tyrosine	(15, 46)
glutamine	(114)	valine	(15, 46)
glycine	(15, 40, 42, 46, 58, 78, 127)	acetamide	(99)
		glucosamine	(100)

In these organotrophs the photosynthetic and chemosynthetic mechanisms may supplement each other under low light intensities (36, 85). Bristol-Roach (23) made measurements of the growth rate of a species of *Scenedesmus* in the light, in the dark with added glucose, and in the light with added glucose. At low light intensities the specific growth rate was 0.15, 0.25 and 0.38, respectively. Thus the rate of growth supported by low light and glucose utilization, operating simultaneously, is experimentally the same as the sum of the growth rates observed independently; i.e., autotrophic and heterotrophic metabolism are additive in supporting growth under these conditions. In *Chlorella* cultures at high light intensities the photosynthetic mechanism was not light-limited and growth was maximum. Addition of glucose did not increase the rate of growth. The significance of the above data is that at levels in a natural system where the photosynthetic mechanism is not light-saturated, algae may be converting organic matter to protoplasm. Rodhe (111), from his observations of ice-covered arctic lakes, has suggested that micro-algae are heterotrophic and are a source of food for the populations of zooplankton occurring in the arctic winter.

ACCESSORY GROWTH FACTORS

A second function of dissolved organic matter is as a source of accessory growth factors. An accessory growth factor may be required by an organism for growth, or it may stimulate growth by supplementing a limiting system already present in the organism. A substance is considered an accessory growth factor if it is necessary in very minute quantities only. This is relative and arbitrary according to the individual making the observations.

That certain algae require accessory growth factors is indicated by the necessity of adding extracts of soil, yeast, etc. in order to culture them in the laboratory (21, 24, 85, 101–103, 110). Rodhe (110) discusses the requirement of organic thermostable and thermolabile substances for growth of certain planktonic algae. This, of course, is only indirect evidence. More direct evidence has been obtained through the use of pure cultures in synthetic media. Table IV lists the growth factor requirements for a number of algae.

If an organism has a requirement for a particular accessory

TABLE IV

GROWTH-FACTOR REQUIREMENTS OF ALGAE

Species	B_1	B_6	B_{12}	Biotin	Histidine	Uracil	Reduced Sulfur Cmpd.	Unknown Org. Fact.	Ref.
CHLOROPHYTA									
Chlamydomonas chlamydogama			R		R				103
Chlamydomonas sp.			R						71
Chlorella sp.								S	71
Lobomonas rostrata			R						71
Coelastrum morus?	R								71
Scenedesmus obliquus		S							11
Scenedesmus quadricauda								S	71
Selenastrum minutum	R								71*
Stichococcus sp.			R						72
EUGLENOPHYTA									
Euglena gracilis			R						108, 109
var. *typica*	R		R						103
var. *bacillaris*	R		R						103
var. *urophora*	R		R						103
pisciformis	R								103
viridis	R		R						103
stellata	R		R						103
CHRYSOPHYTA									
Achnanthes microcephala								S	66
Amphora perspusilla		S	R			S			59
Ditylum brightwellii						R			51
Monochrysis lutheri	S		R						29
Navicula pelliculosa							R		69
Nitzschia acicularis								S	59
Nitzschia palea?								S	59
Prymnesium parvum	S		R						29
Skeletonema costatum			R						30
Syracosphaera carterae								R	103
Syracosphaera elongata	S		R						29
Porteriochromonas stipitata	R		R	R	R				60, 70
PYRROPHYTA									
Cryptomonas ovata var. *palustris*			R						103
Cyanophora paradoxa			R						103
Gymnodinium splendens			R						103
Gymnodinium sp.			R						103
Ochromonas malhamensis	R		R	R	R				60, 87, 121
Paridinium sp.	R		R						103
Synura sp.			R						103

R = required.
S = stimulates.
* Six other cultures of *Selenastrum minutum* or related forms were thiamine independent.

growth factor in culture, it seems reasonable to expect that it will also have this requirement under natural conditions. The above, then, gives presumptive evidence for the presence of growth factors in natural waters. Direct evidence has been obtained by observation of a fluctuation of B_{12} content of a small pool (108) and a thiamine cycle in a pond (54). Also niacin and biotin have been detected in natural waters (54, 56). The presence of these vitamins in natural waters gives hope that further investigation will demonstrate the presence of many other accessory growth factors in natural systems of water.

TOXINS AND ANTIBIOTICS

The third function of dissolved organic matter is to act as a toxin or antibiotic. Before presenting evidence of this specific activity of certain organic compounds, the possibility of their having a dual nature should be mentioned (16, 76); i.e., in low concentrations a compound may act as a stimulant to growth and at high concentrations it may act as an inhibitor, or a particular compound may inhibit one species and stimulate another.

Akehurst (1) observed the seasonal distribution of algae in several small ponds. He defines a toxin as "an excretion product or products of undefined chemical constitution which may also serve as an accessory food and may inhibit or stimulate growth". From his observations he established that there was a cycle of succession of "oil" (Bacillariales, Chrysophyceae, Dinophyceae, Heterokontae, Chloromonadales) and "starch" (Isokontae, Euglininae, Cryptophyceae, Myxophyceae) groups, and postulated that the dominant alga of a predominantly "oil" group population excretes a toxin which inhibits its own growth, may stimulate or inhibit genera of the same or other classes of the oil group, and serves as an accessory growth food for the "starch" group. The same phenomena occur in the "starch" group as related to the "oil" group. Thus, here is presented in theory a mechanism for the succession of algal species in natural systems.

Chlorella has been shown to produce a substance, chlorellin, which in exceedingly low concentrations seems to exert a stimulatory effect on the growth of *Chlorella* and in higher concentrations definitely acts as as an inhibitor to growth (94, 95).

Lefevre et al. (66) discuss the fact that a species of alga seems

to produce several substances active toward other species, and it is the relative proportion of these active substances that determine their overall effect. The complex of active substances may be stimulatory or inhibitory, depending upon the species of the respective alga.

Some evidence of the production by specific organisms of a substance whose activity is specifically toxic has been obtained. *Gonyaulax catenella* synthesizes a poison which is approximately ten times as potent as strychnine when tested on mice. The poison has little effect in fish, but death of humans has been attributed to eating shellfish which collect *Gonyaulax* in the gut (25). *G. tamerensis* poisonings have also been reported from Nova Scotia (25). *Gymnodinium brevis* has been associated with the mass death of marine organisms off the west coast of Florida (27, 45).

Cultures of *Chlorella* produce a substance which is toxic, as discussed above (94, 95). *C. vulgaris* and *C. pyrenoidosa* produce chlorellin which possesses antibacterial properties toward both Gram-positive and Gram-negative bacteria. *Microcystis* does the same (68). Flint and Moreland (34) have found exudates from blue-green algae to be lethal to other algae. Nielsen (91) has evidence for antibiotic produced by *Chlorella pyrenoidosa* and *Thalassiosira nana*. *Chlorella vulgaris* and *Nitzschia frustulum* secrete substances inhibitory toward each other (105). *Pandorina* secretes a substance inhibiting *Chlorella* and *Nitzschia* (105).

Prymnesium parvum, a free-living brackish water phytoflagellate of the Chrysophyceae, produces an extra-cellular toxin which kills fish or other organisms which breathe by means of gills (117, 118).

Several blue-green algae which produce toxic materials differ from *Prymnesium* in that the toxins are intracellular and are released only on death and decay or ingestion by other organisms (62, 97, 115). Sheep, horses, dogs, pigs, cattle, chickens, turkeys, ducks, geese and rabbits have been killed by drinking water which contains high populations of algae. Test animals, guinea pigs, mice and cats, given subcutaneous or intraperitoneal injections, were killed. Injections of pond water from which the algae had been filtered did not affect the animals. The genera of Cyanophyta which appear to possess toxic species are *Microcystis, Anabaena, Aphanizomenon, Nodularia* and *Coelosphaerium*.

Lefevre et al. (66–68) have performed some very interesting

experiments, in both field and laboratory, related to the effect of certain species of algae on other species of algae. They found that algae produce substances in culture medium which may be algastatic, algacidal or algadynamic. Substances analogous to chlorellin from *Chlorella* were obtained: phormidine, pandorinine and scenedesmine. Lefevre proposes that in natural waters, when for reasons not known a species of alga multiplies abundantly, the active substances it secretes for the most part inhibit the development of other algae present which eventually die. A very small number of individuals in every species is resistant. As soon as the dominant alga disappears, the antibiotic action ceases and the resistant individuals commence an active multiplication.

Some evidence for antagonistic activity of algal populations toward microcrustaceans has been obtained. Lucas (73) experimented with *Nitzschia closterium* and the zooplankters *Neomysis vulgaris* and *Eurytemora hirundoides*. His evidence gives some indication of an excluding effect of *Nitzschia*, but is not conclusive. Bainbridge (14) obtained variable results, indicating that there is an antagonistic effect of algae on zooplankton, but this effect is probably a special case. Ryther (113) was able to demonstrate that the filtering rate of *Daphnia* is inhibited by substances produced by *Chlorella vulgaris*, *Scenedesmus quadricauda* and *Navicula pelliculosa*. The inhibitory product of *Chlorella* appears to be identical with chlorellin. The effect of the inhibitor is mediated partially by a substance in the water, but a much more pronounced effect seems to be produced by release of the substance from ingested cells within the animal.

CHARACTERIZATION OF TOXINS

Three of the toxins produced by algae have been partially characterized. Chlorellin diffuses through a collodion membrane, and thus its molecules are probably less than 15 Å units in diameter. It is soluble in 95 per cent alcohol, ether and petroleum ether, and is more readily extracted from aqueous alkaline solution than from acid solution. From this it is concluded that the active portion is an organic base. It is destroyed by heat but probably not by oxidation. It checked growth more at higher pH than at lower pH, indicating that it is more effective in the basic than the salt form (94).

Early attempts to characterize the toxin produced by *Microcystis* were not conclusive (62). This toxin is easily soluble in water. In neutral solution it is thermostable but it is alkali labile. It diffuses slowly through a cellophane membrane, and purified fractions fail to produce an antibody formation (116). It is a rapidly acting cytotoxic agent with particular tendency to damage hepatic parenchymal cells in the rat, and it leads to cellular degeneration and necrosis in other organs, particularly heart and kidneys. It resembles closely the thermostable toxin of the mushroom *Amanita phalloides* with respect to pathological effects, chemical properties and composition (13).

The toxin produced by *Prymnesium parvum* extracellularly is thermolabile and also may be destroyed by oxidation. It does not diffuse through cellophane tubing. Its activity is constant above pH 7.5, and zero at pH 6.0. The effect of pH is reversible, and this is similar to the effect of pH on some enzyme proteins. It is readily absorbed by various materials and can be destroyed by bacterial action. Its effects are upon those organisms which depend upon gills for breathing (117, 118).

CHELATION

The fourth possible function of dissolved organic matter is the ability to influence the availability of trace metals by chelation, i.e., the formation of a very stable ring complex between an organic compound and a metallic element (2, 3, 82). Trace metals are necessary as co-factors of enzyme systems and as components of other biologically significant metallo-organic compounds; e.g., zinc is a constituent of carbonic anhydrase, cobalt is present in vitamin B_{12}, copper replaces iron in the blood pigment of certain molluscs and is also present in several enzyme systems, copper and manganese are essential to the development of chlorophyll, and iron is necessary to the cytochromes and in hemoglobin.

Chelation of trace metals may affect algae in four ways:

a) The organic chelate may affect the availability of a trace metal ion by lowering its effective concentration in the water system below the level of requirement by a particular organism. Thus a trace metal required for a particular biological system may become completely unavailable or only partially available but limiting to the normal growth and reproduction of the organism.

b) If there is an excess of a trace metal which is toxic to a particular organism, chelation may reduce the metal ion concentration below the level of toxicity to that organism.

c) Chelation may remove a metal ion which is antagonistic to a metal poison, effectively increasing the relative concentration of the poison to a level at which it becomes toxic.

d) If a trace metal tends to precipitate out and become essentially unavailable to an organism, chelation may maintain that particular element complexed in a soluble state and thus keep the effective level of concentration above that which would prevail if chelation did not occur.

The extent to which the availability of a trace metal is affected by chelation depends on the amount and nature of the chelating agents present, whether there is selective chelation, the position of the metal in the Mellor-Maley series (activity series), the relative concentration of the metal in the medium, and the hydrogen-ion concentration.

The ionic balance within a cell is probably different from that outside the cell, and the effect of a chelating agent may also depend upon whether or not it can penetrate the cell membrane. A chelating agent which can not penetrate the cell membrane may exchange metals with a chelating agent in the membrane or may lose its metal ion to a more effective chelating agent in the membrane. The chelating agent may also set up an effective gradient and trap metal ions as they pass out of the cell to the external medium. If a chelating agent is able to penetrate the cell membrane, it may be distributed to parts of the cell where it competes with enzymes for metals which are necessary to the enzyme activity (119, 120, 130). If a metal normally can not penetrate a cell membrane, a chelate complex may be able to do so. This may be the manner in which normally insoluble ferric ion is made available to algal organisms.

It is known that chelating agents affect the growth performance of various organisms in culture (58, 61, 103, 126), and it has been suggested that chelation may be an important factor in natural systems (35, 37, 51, 103). An example of a possible action of chelating agents may be made in reference to the iron cycle in natural waters (112). In neutral or alkaline waters in which there is also some oxygen present, iron will be in the ferric state and

precipitate as the hydroxide or phosphate. This precipitate will settle from the photic zone, and algal organisms will be deprived of a source of iron. Now, if there are organic compounds present capable of chelating iron, the iron will remain in an effectively soluble state in the photic zone and thus will be available to the organisms.

It has been demonstrated that certain amino acids, peptides and pigments are present in natural bodies of water. It is also known that amino acids and porphyrins (moiety of chlorophylls) are effective chelating agents. Amino acids are not capable of chelating ferric ion (2), but there are other organic compounds known to do so. Thus it appears that the potentiality, at least, for organic matter influencing the availability of trace metals is present in natural bodies of water. Resolution of this difficult question can come only through laboratory investigations of the trace metal requirements of pure cultures of algae and field studies of trace metal cycles, dissolved organic cycles, and the succession of algal populations.

ECOLOGICAL IMPLICATIONS OF ORGANIC MATTER

In the above discussion the possible functions of organic matter in natural systems have been emphasized rather heavily. One should be reminded that, although the role of organic matter may be significant, it is only a small part of the total number of environmental factors which may influence the distribution of living organisms in time and space.

In the course of evolution, algal and/or protozoan forms have developed which run the gamut from completely autotrophic through auxotrophic (requiring only accessory growth factors), to completely heterotrophic activities, with all degrees and stages in between. There are examples of auxotrophs which require only one accessory growth factor. An organism such as *Ochromonas malhamensis* approaches very closely to the heterotrophic end of the scale. It is able to maintain itself marginally by use of the photosynthetic mechanism alone but grows much more readily when supplied a complex mixture of organic materials (87). Rodhe (111) has suggested that microalgae in arctic lakes have a heterotrophic form of nutrition during the winter under ice cover and thus may form populations large enough to support large

populations of zooplankton at this time of year. Thus it is apparent that one may expect all degrees of activity in obtaining energy and nutrients for growth. The greater the photosynthetic mechanism is reduced in activity, the more the organism may have to depend upon heterotrophy to maintain itself or to compete with other organisms. The greater the number of steps in the synthetic pathways which are blocked by lack of ability to produce an enzyme, the greater the number of exogenous organic compounds required and the greater the approach to heterotrophy.

It has been demonstrated in theory and in some cases in reality that organic matter may function in at least four ways in influencing living organisms: as a substrate, as a source of accessory growth factors, as a source of toxins, and as a complexing agent of trace metals. What are the ecological implications of these functions?

The first and most fundamental implication involves the basic nutrition and physiology of algal organisms, and, in addition, bacteria, fungi and aquatic plants. A particular organism may have a dependency on organic matter in terms of any one or all four of the proposed functions of organic matter. The requirements may be simple or very complex. In addition, each requirement has a range within which the organism is satisfied. If the environmental condition of the requirement is at either extreme of the range, the organism grows poorly. If the condition is optimal, the organism grows very well indeed. Thus, in a broad sense, organic matter affects living organisms because not only must the organism be present in a system but the organic matter must be present within a range which allows the organism to grow and reproduce.

The second implication is more specific. It involves the succession of organisms in time and space. The amount and nature of the organic matter present in a natural system are dependent upon its sources as discussed before. This quantity in nature fluctuates with the seasons. Not only must an organism be present but it must be present at a particular time and in a particular space where, other environmental factors being favorable, the organic factors are within the range of requirements of the particular organism. The more optimal the conditions, the greater the chance for the organism to be successful. Thus a particular species may

be successful in the littoral zone and not in the open water, or vice versa. It may be successful at a particular time of year and not at any other because of some fluctuation in accessory growth factor content of the water, a restriction on the necessary trace metal, or perhaps due to production of a substance by some other organism which is stimulatory or toxic to its metabolism.

A third implication is even more specific and involves the effects on the spatial distribution of other organisms, including zooplankton, fishes and perhaps bacteria. This may apply in particular to Hardy's Animal Exclusion Theory (14, 48, 49, 73, 113) as opposed to the Grazing Theory proposed by Harvey and discussed by several authors (14, 33, 90, 107).

Hardy found an inverse relation between density of phytoplankton and zooplankton. He postulated that in many cases this relationship could be explained upon an excluding effect due presumably to chemical agents produced by the plants which were irritating or antagonistic to the animal organisms. The zooplankton were believed capable of migrating only vertically and remained in the phytoplankton areas for a time inversely proportional to the density of the phytoplankton. Upon migrating downward out of the phytoplankton patches, the zooplankton are transported by subsurface currents to areas in which phytoplankton are less dense. Upon migrating upward into these less dense patches, the zooplankton remain here for a longer period of time and thus tend to become concentrated in areas of low phytoplankton population.

There seems to be evidence that, at least in very special cases, organic metabolites may have an antagonistic effect toward fish and lower animals because of toxins secreted or released during autolysis by certain phytoplankton organisms. Thus there is some manner of support to Hardy's Animal Exclusion Theory, and a specific ecological effect of organic metabolites.

FATA VIAM INVENIENT

The foregoing discussion has been extremely speculative. Conclusions regarding the role of organic matter in natural bodies of water have been arrived at mainly through inference or analogy, and much has been taken from marine work. The limnologist has attempted to explain the distribution and interrelationships of freshwater organisms by attacking the problem from the more conven-

tional physical and chemical points of view. The results have been incomplete. The biochemist and physiologist are just beginning to penetrate the frontiers of understanding the basic metabolic pathways of synthesis and degradation within living organisms. That micro-quantities of both organic and inorganic matter are significant to the basic growth, behavior and success of living organisms is apparent. By using much of the new information from biochemistry and by use of new tools and techniques, such as paper chromatography, chelate complexes, radioactive tracers and microbiological assay as they are developed, the limnologist may be able eventually to complete the picture he is trying to paint. Johnston (63) has discussed some of these techniques. The attack may be initiated by the use of pure cultures in the laboratory to determine the basic requirements of the more significant algal forms in terms of their ability to utilize various organic substrates, their requirement for growth factors, their reaction toward antibiotics, and their requirement for trace metals. In the field he may be able to determine the yearly cycles of specific organic nutrients, vitamins and trace metals. Many questions may be answered by correlating these laboratory and field studies with analyses of populations and their succession in nature. Some investigators have already recognized the utility of these new methods and have begun researches along this line. Vallentyne (123) has recently published a short paper entitled "Biochemical Limnology". These tools should be considered and used by the limnologist to usher in what might be a new era in limnology and in the investigation of the interrelations of dissolved organic matter, phytoplankton, and zooplankton.

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